

# Analysis of Polyamines in Higher Plants by High Performance Liquid Chromatography<sup>1</sup>

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## ABSTRACT

A sensitive (0.01–1 nmol) method has been developed for the analysis of polyamines in higher plant extracts based on high performance liquid chromatography (HPLC) of their benzoyl derivatives (Redmond, Tseng 1979 J Chromatogr 170: 479–481). Putrescine, cadaverine, agmatine, spermidine, spermine, and the less common polyamines nor-spermidine and homospermidine can be completely resolved by reverse phase HPLC, isocratic elution with methanol:water (64%, v/v) through a 5- $\mu$ m C<sub>18</sub> column, and detection at 254 nm. The method can be directly applied to crude plant extracts, and it is not subject to interference by carbohydrates and phenolics. A good quantitative correlation was found between HPLC analysis of benzoylpolyamines and thin layer chromatography of their dansyl derivatives. With the HPLC method, polyamine titers have been reproducibly estimated for various organs of amaranth, *Lemna*, oat, pea, *Pharbitis*, and potato. The analyses correlate well with results of thin layer chromatography determinations.

The widespread occurrence of Put,<sup>2</sup> Spd, and Spm in bacterial, animal, and plant cells is now well established (6, 10, 26). In recent years, research on these polyamines and their less common homologs (Dap and Cad) of higher plants (26), algae, and bacteria (1, 9) has focused on the suggestive evidence (6, 10, 19) for their involvement in the regulation of growth and development. For example, exogenously added polyamines promote callus formation in Jerusalem artichoke tuber explants (3), prevent leaf senescence (15), and promote macromolecular synthesis and mitosis in oat leaf protoplasts (14). The polyamine biosynthetic enzyme arginine decarboxylase is regulated by phytochrome (7) and GA<sub>3</sub> (8) in pea buds and internodes. The evidence accumulated so far needs to be supported by detailed analysis of the endogenous polyamine pools and *de novo* synthesis from labeled precursors during the developmental processes these compounds seem to affect.

The most sensitive and widely used method of polyamine analysis is based on the dansylation of the amino groups under alkaline conditions, separation of the dansyl derivatives by TLC, and quantification by fluorimetry (22, 25). Although originally developed for the analysis of animal and bacterial polyamines, the dansyl method has been adapted for use with higher plant extracts

(27, 28). HPLC analysis of dansyl-polyamines has also been reported (23, 24). The dansyl reagent is not specific for amino groups, and the presence of carbohydrates and phenolics in plant cells causes substantial interference and reduces the accuracy of quantitative analyses.

A recent report (20) showed the separation of polyamine standards (Put, Cad, Spd, Spm) based on the Schotten-Baumann benzoylation reaction, and subsequent analysis by reverse-phase HPLC. We have extended the range of compounds that can be resolved by this procedure and developed the method for an accurate estimation of polyamine titer in a variety of crude higher plant extracts. These results are reported below, and the correlation with the analysis of dansyl-polyamines is discussed.

## MATERIALS AND METHODS

**Plant Material.** The following plant material was grown in controlled environment growth rooms, in plastic pots containing washed vermiculite, subirrigated twice daily with a 1.2 g/L solution of Hyponex (Hydroponics Chemicals Co., Copley, OH): grain and vegetable amaranth (*Amaranthus cruentus*, *A. hypochondriacus*; *A. tricolor*); oat (*Avena sativa* cv. Victory); *Pharbitis nil*; and bean (*Phaseolus vulgaris* cv. Taylor). Plants were grown under a 16-h photoperiod, 25°C, with a 9:1 energy mixture of fluorescent and incandescent light at an energy level of about 17,600 ergs cm<sup>-2</sup> sec<sup>-1</sup>. Cultures of *Lemna gibba* were a gift from Dr. C. F. Cleland; both fresh cultures and lyophilized samples were analyzed with similar results. Peas (*Pisum sativum* cv. Alaska) were grown in darkness at 26°C and 70% RH. Potato tubers (*Solanum tuberosum* cv. Russet Burbank) were obtained from a local market and kept stored at 5°C until use. Tissues were extracted in 5% cold HClO<sub>4</sub> at a ratio of about 100 mg/ml HClO<sub>4</sub>. After extraction for 1 h in an ice bath, samples were pelleted at 48,000g × 20 min, and the supernatant phase, containing the 'free' polyamine fraction, was stored frozen at -20°C in plastic vials. HClO<sub>4</sub> extracts were stable for polyamine analysis by TLC or HPLC for more than 6 months under these conditions, provided excessive refreezing and thawing were avoided. Since polyamines have been shown to bind to glass (18; SS Cohen, personal communication), care was taken, for both plant extracts and standards (see below), to store samples in plastic vials.

**Chemicals.** The following polyamines were obtained as their hydrochlorides (Sigma): Dap, Put, Cad, Spd, and Spm. Agm (Sigma) was obtained as its sulfate. The hydrochlorides of Homo-Spd and APCad were gifts from Prof. Seymour S. Cohen (SUNY, Stony Brook, NY). Nor-Spd was used as the free base (Aldrich). One-mM stocks in 0.01 N HCl of these compounds were stored as above, and were stable for at least 6 months at -20°C. Benzoyl chloride (Baker) and dansyl chloride (Sigma) were reagent grade, >95% purity.

**Dansylation and TLC Analysis.** The method of Seiler and Wiechmann (25) as adapted for plant tissues (28) was used with some modifications as follows. Two hundred  $\mu$ l of HClO<sub>4</sub> extract

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<sup>2</sup> Abbreviations: Put, putrescine; Spd, spermidine; Spm, spermine; Dap, diaminopropane; Cad, cadaverine; Agm, agmatine; Homo-Spd, homospermidine; APCad, aminopropylcadaverine; Nor-Spd, norspermidine; dansyl, 1-dimethylamino-5-naphthalenesulfonyl; a.u.f.s., absorbance units full scale.

Table I. *Chromatographic Parameters for the Separation of Benzoyl-Polyamines by Reverse-Phase HPLC*  
 Separations carried out at room temperature. The flow rate was 1 ml/min. The  $t_0$  was 2.58. The theoretical plate count ( $n$ ) was 7,900 at 64% methanol and 9,600 at 60% methanol.

Polyamine	64% Methanol				60% Methanol			
	Retention time	$k'$	$\alpha$	$R_s$	Retention time	$k'$	$\alpha$	$R_s$
	min				min			
Dap	5.84 $\pm$ 0.01 <sup>a</sup>	1.26	1.07	0.82	7.49 $\pm$ 0.06 <sup>a</sup>	1.90	1.07	2.22
Put	6.12 $\pm$ 0.02	1.35	1.19	2.19	7.85 $\pm$ 0.05	2.04	1.25	3.50
Cad	6.81 $\pm$ 0.03	1.61	1.31	3.59	9.18 $\pm$ 0.05	2.56	1.18	2.81
Agm	8.12 $\pm$ 0.03	2.11	1.11	1.54	10.36 $\pm$ 0.04	3.02	1.36	5.22
Nor-Spd	8.72 $\pm$ 0.06	2.34	1.16	2.24	13.18 $\pm$ 0.11	4.11	1.19	3.26
Spd	9.73 $\pm$ 0.08	2.72	1.20	3.12	15.31 $\pm$ 0.12	4.91	1.25	4.21
Homo-Spd	11.13 $\pm$ 0.01	3.26	1.03	0.50	18.30 $\pm$ 0.12	6.12	1.04	0.81
APCad	11.49 $\pm$ 0.11	3.36	1.51	6.2	19.05 $\pm$ 0.13	6.39	1.66	8.91
Spm	15.82 $\pm$ 0.20	5.06			29.99 $\pm$ 0.34	10.63		

<sup>a</sup>  $\pm$ SE.

were mixed with 400  $\mu$ l of dansyl chloride (5 mg/ml in acetone, prepared fresh), and 200  $\mu$ l of saturated sodium carbonate were added. After brief vortexing, the mixture was incubated in darkness at room temperature overnight. Excess dansyl reagent was removed by reaction with 100  $\mu$ l (100 mg/ml) of added proline, and incubation for 30 min. Dansylpolyamines were extracted in 0.5 ml benzene, and vortexed for 30 s. The organic phase was collected and stored in glass vials at  $-20^\circ\text{C}$ . Dansylated extracts were stable for up to 1 month. Standards were processed in the same way, and 20 nmol were dansylated for each, alone or in combination. TLC was performed on high resolution LK6D silica gel plates (Whatman). Up to 50  $\mu$ l of dansylated extract were loaded on the pre-adsorbent zone, and the chromatogram was developed for about 1 h with either of two solvent systems: chloroform:triethylamine (25:2, v/v) or cyclohexane:ethylacetate (5:4, v/v). Identification of the unknowns was done by comparison of the  $R_f$ s in these two solvent systems, fluorescence spectra, and at least partially by mass spectrometric analysis. Results have been consistent and reproducible for all our plant extracts. After TLC, the dansylpolyamine bands were scraped, eluted in 2 ml ethylacetate, and quantified with an Aminco-Bowman spectrophotofluorimeter, with excitation at 350 nm and emission at 495 nm. Analysis of the dansyl derivatives had to be performed immediately because of the rapid fading of fluorescence after TLC (22, 25). Fluorescence was somewhat more stable following TLC in chloroform:triethylamine.

**Benzoylation and HPLC Analysis.** Standards and plant extracts were benzoylated according to Redmond and Tseng (20). One ml 2 N NaOH was mixed with 250 to 500  $\mu$ l of  $\text{HClO}_4$  extract. After addition of 10  $\mu$ l benzoyl chloride, vortexing for 10 s, and incubation for 20 min at room temperature, we added 2 ml saturated NaCl. Benzoyl-polyamines were extracted in 2 ml diethyl ether (anhydrous grade; Baker). After centrifugation at  $1500g \times 5$  min, 1 ml of the ether phase was collected, evaporated to dryness under a stream of warm air, and redissolved in 100  $\mu$ l methanol (Baker; HPLC grade). Standards were treated in a similar way, with up to 50 nmol of each polyamine in the reaction mixture. Except occasionally for Agm, the benzoylation of any one standard did not seem to be affected by the presence of a large excess of the other polyamines. The benzoylated samples were stored at  $-20^\circ\text{C}$ . Except for Agm and Spd, the benzoylpolyamines were stable under these conditions for several months. The absorbance (254 nm) of benzoyl-Agm and benzoyl-Spd tended to increase when stored beyond 2 weeks, presumably due to decomposition products. Benzoyl-Spd showed a split peak after HPLC in old samples. HPLC analysis was done with a programmable Altex-Beckman model 322 liquid chromatograph. The solvent system consisted of methanol:water, run isocratically at 60 to 65% methanol, at a flow

rate of 1 ml/min. Preliminary experiments showed that quantification was not as precise when a solvent gradient was programmed, and no significant gain in resolution was obtained. Acetonitrile:water (52%, v/v) was also found to be more sensitive, and useful for rapid separations not involving Agm (Fig. 2). The benzoylated extracts were eluted at room temperature through a  $4.6 \times 250$  mm, 5- $\mu$ m particle size reverse-phase ( $\text{C}_{18}$ ) column (Altex-octadecylsilane) and detected at 254 nm. Sensitivity was routinely set at 0.04 a.u.f.s., although the response was found to be linear down to 0.005 a.u.f.s. The results were quantified with a 3390A Hewlett-Packard integrator. One- and two-point calibrations were found sufficiently accurate for standards (Table I) since the injection loop used was fixed volume (20  $\mu$ l). The following chromatographic data were calculated on the various benzoyl-polyamine standards, at two different solvent strengths (selectivity essentially unchanged) (see Table I):

$$k' = \frac{t_r - t_0}{t_0} \quad \alpha = \frac{k'_2}{k'_1} \quad R_s = \frac{1/4 \cdot (\alpha - 1)}{\alpha} \sqrt{N} \cdot \left( \frac{k'}{1 + k'} \right)$$

where  $k'$  is the capacity factor,  $t_0$  is the retention time of unretained solute,  $t_r$  is the retention time of benzoyl-standard,  $\alpha$  is the relative retention,  $R_s$  is the resolution of two peaks, and  $N$  is the theoretical plate count (7,900 at 64% methanol; 9,600 at 60% methanol) (26). Statistical analyses on the HPLC and TLC results were performed with the PROPHET system, a national computer facility of the Chemical/Biological Information Handling Program of the National Institutes of Health.

## RESULTS AND DISCUSSION

The benzoylpolyamines are separable under a variety of conditions through reverse-phase HPLC (Table I; Figs. 1 and 2). A typical chromatogram run at 64% methanol is shown in Figure 1. All peaks shown could be fully resolved in less than 20 min. Since Nor-Spd and Homo-Spd were absent from our samples (Table II), the solvent strength could be further increased (up to 66–68% methanol) without significant loss in resolution or reproducibility so as to give separation times of 12 to 15 min for complete elution of Spm, the final peak. From the wide range of solvent strengths surveyed, isocratic elution with 60 and 64% methanol have proved useful for routine purposes. Chromatographic parameters for HPLC of benzoyl standards are shown in Table I. Retention times were extremely reproducible, provided the flow rate was kept constant and clean samples were injected. The standard deviation for retention times varied from 0.4% (Dap) to 2.5% (Spm). Optimal values for the capacity factor ( $k'$ ) should range within 1 to 10 (29) and this criterion was met in both solvent strengths (Table I). However, the  $R_s$  between Dap-Put and Homo-Spd-APCad are

Table II. Polyamine Levels in Higher Plant Tissues as Determined by HPLC of Their Benzoylated Derivatives

Species	Organ	Polyamine					
		Dap	Put	Cad	Agm	Spd	Spm
nmol/g fresh wt							
<i>Amaranthus cruentus</i> (grain amaranth)	Cotyledon (7 d)	Trace	43	ND	275	291	47
	Cotyledon (21 d)	Trace	5	ND	41	6	16
<i>A. hypochondriacus</i> (grain amaranth)	1° Leaf (12 d)	Trace	23	ND	306	363	51
	1° Leaf (21 d)	Trace	7	ND	51	52	16
<i>Avena sativa</i> (oat)	1° Leaf (7 d)	11.5	41	ND	95	147	21
	Mesophyll protoplasts <sup>a</sup>	1.3	22	ND	9	8	3
<i>Lemna gibba</i> (duckweed)	Vegetative fronds	ND <sup>b</sup>	7	ND	439	570	Trace
	Flowering fronds	ND	16	ND	503	487	41
<i>Pharbitis nil</i> (Japanese morning glory)	Cotyledon (11 d)	ND	Trace	ND	439	112	Trace
	1° Leaf (11 d)	ND	Trace	ND	698	409	Trace
	Apex (11 d)	ND	Trace	ND	114	209	Trace
<i>Pisum sativum</i> (pea)	3° Internode (6 d) <sup>c</sup>	ND	2,570	546	1,195	835	ND
	Bud (6 d)	ND	217	ND	1,650	1,500	66

<sup>a</sup> Values expressed as nmol/10<sup>6</sup> protoplasts. Yields were usually 9 to 10 × 10<sup>6</sup> protoplasts/g leaf.

<sup>b</sup> Not detectable.

<sup>c</sup> Extracts from etiolated seedlings. In all other cases, polyamines were extracted from light-grown seedlings at the indicated age.

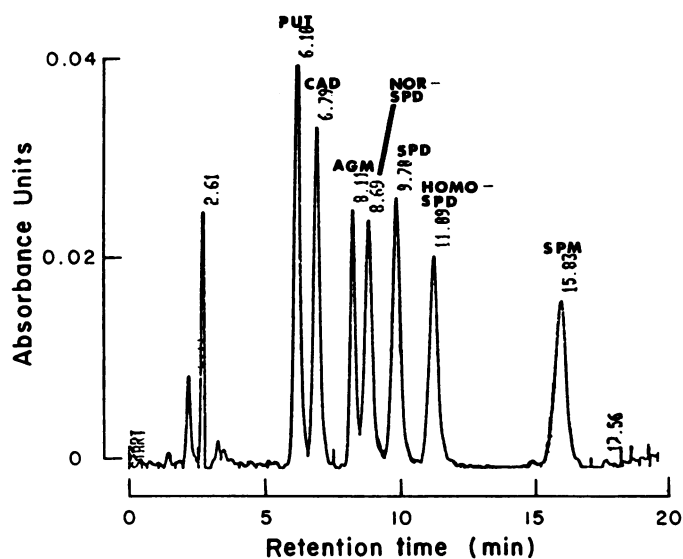


FIG. 1. HPLC of benzoylated polyamine standards. Solvent system: 64% methanol. Absorbance at 254 nm. One nmol of each standard.

below the acceptable limits ( $R_s = 1$ ) when eluted with 64% methanol, and the peak overlap is sufficiently high to obscure the quantitative estimations.  $R_s$  in both cases was improved by a simple change in solvent strength, and this allowed for precise estimation of Dap and Put in cereal samples (Table II). The resolution of the common polyamines from their less common homologs is better than that found for TLC and comparable to that obtained with the HPLC systems available (1), with a reduction in analysis time.

The usual range of sensitivity for our analyses was 0.1 to 1 nmol (Figs. 1, 3, 4, 6, and 7), similar to that reported with the dansyl method (23, 25). This could be extended if acetonitrile:water was used (52%, v/v) instead, to about 10 pmol for Put, Cad, Spd, and Spm (Fig. 2). Detection limits of 50 pmol were obtained with the methanol:water system for standards and plant samples. The

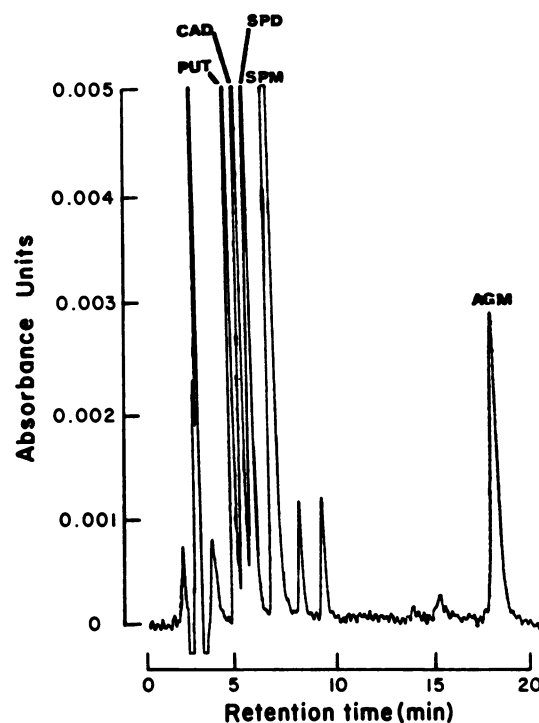


FIG. 2. HPLC of benzoyl polyamine standards. Solvent system: 52% acetonitrile. Absorbance at 254 nm. 0.25 nmol of each standard.

above conditions for separation of benzoyl polyamines are based on the accepted mechanism for reverse-phase HPLC, in which the resolution of a mixture depends on the solvophobic interaction between the sample components and the non-polar hydrocarbon groups attached to the silica gel matrix. Since it has been shown recently (4) that the residual hydrophilic silanol groups of the stationary phase affect resolution significantly, the separations reported here are susceptible to further improvement provided the residual silanols are masked by an appropriate reagent.

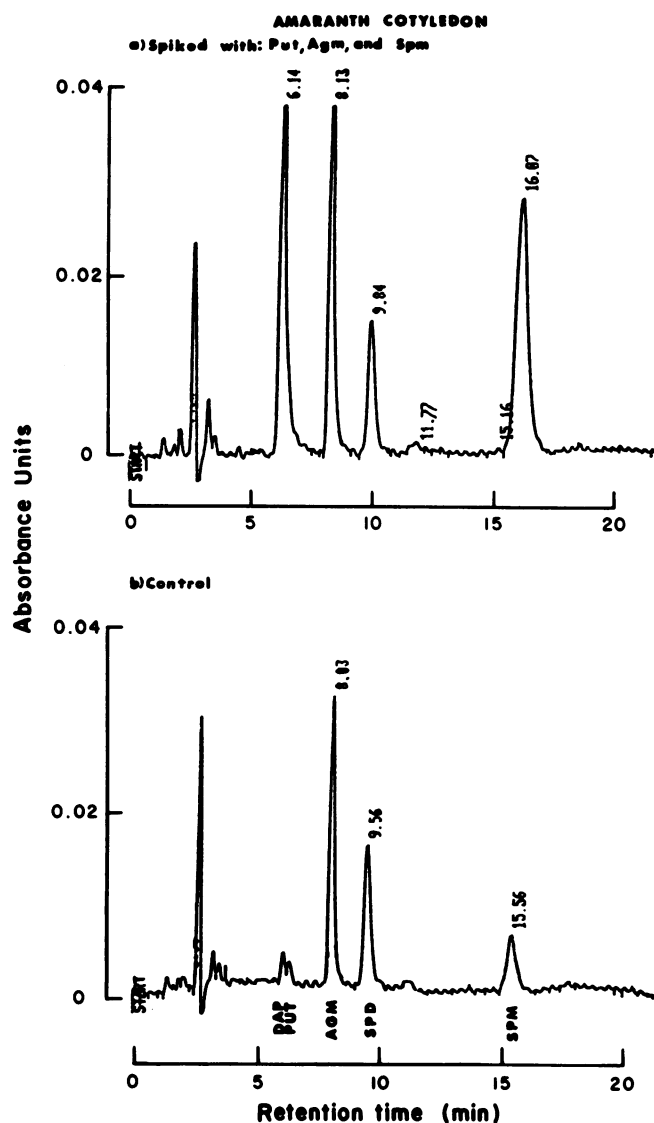


FIG. 3. Identification of polyamines in amaranth cotyledon extracts by spiking with 1.0 nmol each of Put, Agm, and Spm. Conditions for separation as in Figure 1.

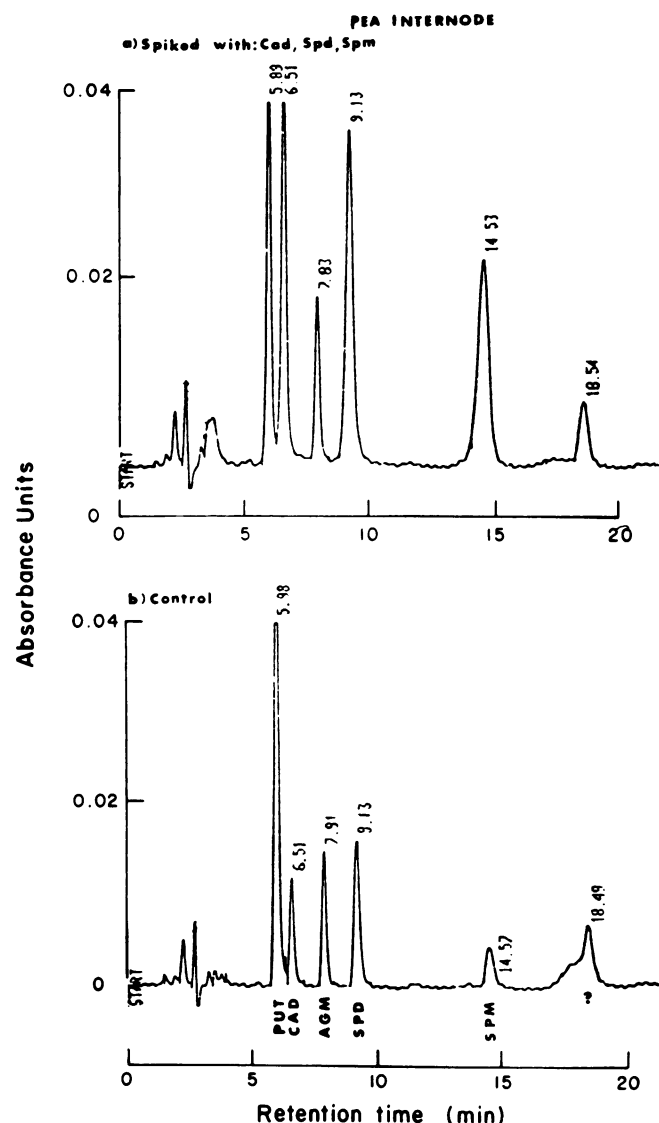


FIG. 4. Identification of polyamines in etiolated pea internode extracts by spiking with 1.0 nmol each of Cad and Spm, and 0.5 nmol of Spd. Solvent system: 65% methanol. Other conditions as in Figure 1.

The highly reproducible retention times (Table I) obtained with HPLC of polyamine standards allowed for preliminary identification of the unknown peaks in plant extracts. In addition, each peak was spiked with known amounts of a particular standard, and the increase in the areas of specific peaks at different solvent strengths (Figs. 3 and 4) confirmed the nature of the unknowns. No variation in elution volume or splitting of the peaks were observed. The correlation of estimates obtained by HPLC and TLC (Fig. 8) further support the nature of the peaks observed in all samples studied in this report. The presence of unknown peaks in amaranth cotyledon (Fig. 3) and pea internode (Fig. 4) extracts has been confirmed, the nature of which is not yet established. Unknown bands with the same fluorescence spectrum of dansyl-polyamines have been detected by TLC (Fig. 5, lanes 3, 4, 6, 7).

The dansylation procedure (25) as adapted for plant samples (27) was used for polyamine analysis with various plant extracts. The common polyamines are well resolved (Fig. 5, lane 5) with the chloroform:triethylamine system. In general, no background interference is found for quantification of leaf and stem samples (Fig. 5, lanes 1–8 and 10). However, in samples with high content of carbohydrates and/or phenolics, as in cell wall digests from oat leaves (Fig. 5, lane 9), accurate estimation of polyamine titers was

not possible. The interference is caused by side products of the dansyl reaction formed in the presence of sugars, ammonia, or amino acids (as for hydrolyzed samples during the analysis of bound polyamines) (22). These samples had to be purified through silica gel (13) before derivatization, with a significant loss in recovery. By contrast,  $\text{HClO}_4$  extracts could be directly analyzed by HPLC after the benzylation procedure, as in the case of potato tuber extracts (Fig. 6), without significant interference from carbohydrates or other compounds.

HPLC of benzoylpolyamines has been used for analysis of a wide range of organs and species of higher plants (Table II; Figs. 6 and 7). A good correlation between estimates made by TLC and HPLC is apparent (Fig. 8) as shown for amaranth and oat extracts analyzed simultaneously through both procedures. Since the  $\text{HClO}_4$  extracts are stable for several months (see "Materials and Methods"), correlation coefficients were above 0.8 even if TLC and HPLC analyses were performed 2 months apart (data not shown). The HPLC method has been used to show the organ-specific polyamine pattern in buds and internodes of etiolated pea seedlings (Table II; Fig. 7). Cadaverine is present only in internodes, cotyledons, and roots of peas (N. Young, personal com-

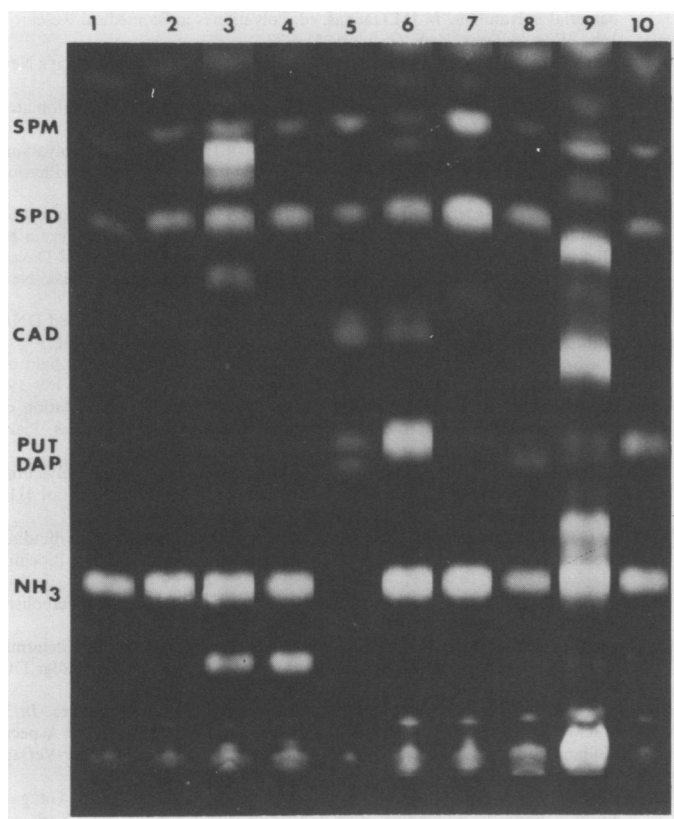


FIG. 5. TLC of dansyl-polyamines in chloroform:triethylamine (25:2, v/v). Lane 1, grain amaranth cotyledon; lane 2, grain amaranth leaf; lane 3, wild type *A. tricolor* leaf; lane 4, white-stemmed *A. tricolor* leaf; lane 5, standards (0.4 nmol each); lane 6, etiolated pea internode; lane 7, etiolated pea bud; lane 8, oat leaf; lane 9, oat leaf cell wall digest; lane 10, bean flower.

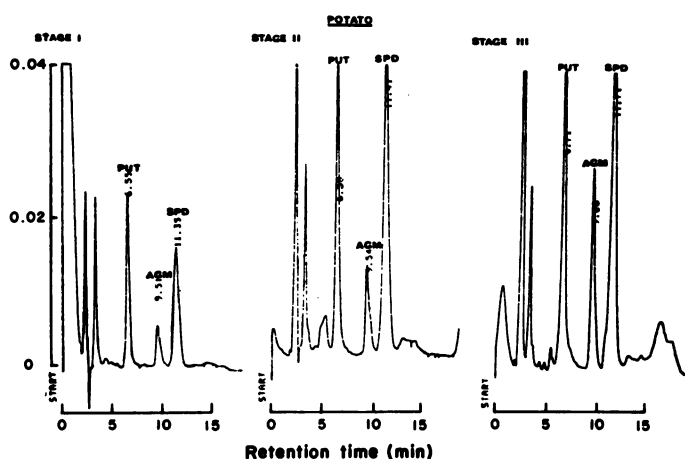


FIG. 6. Polyamines in sprouting potato tuber. Stage I, dormant bud; stage II, early sprouting; stage III, advanced sprouting. Solvent system: 62% methanol. Other conditions as in Figure 1. Equal fresh wt equivalent per sample.

munication), and in some other members of the *Leguminosae* (26). The findings in pea have been related to the phytochrome and hormonal control of arginine decarboxylase activity (7, 8) and extended to polyamine titer studies in relation to red light inhibition or promotion of growth (12). The striking increases in Put, Agm, and Spd in potato tubers during sprouting (Fig. 6) have been correlated with growth and increased ornithine decarboxylase activity (17). Also apparent are the large decreases in poly-

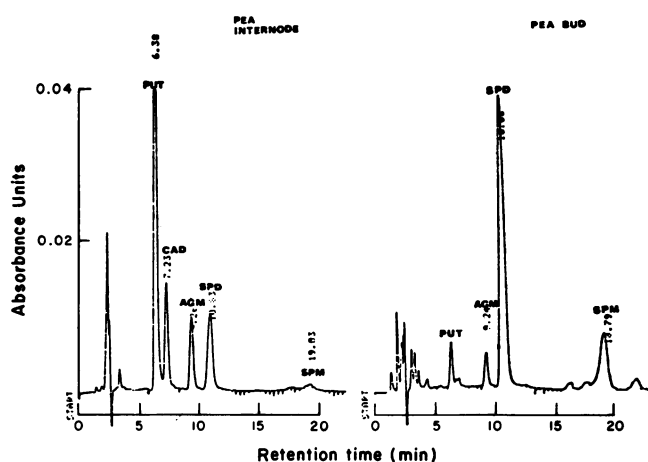


FIG. 7. Polyamines in bud and internode of etiolated pea seedlings. Conditions for separations as in Figure 6.

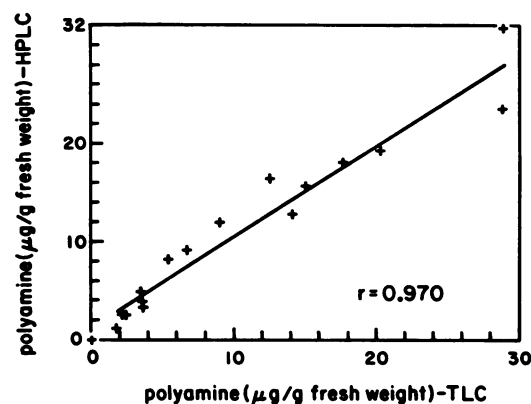


FIG. 8. Correlation for polyamine titers obtained by TLC analysis of dansylated and HPLC analysis of benzoylated polyamines. Conditions for analysis as in "Materials and Methods."

amine titer in amaranth cotyledons and oat leaves during aging *in situ* (Table II), which are consistent with findings in oat leaves during senescence *in situ* and *in vitro* (17). Titrers are high in rapidly growing organs, as in the apex of *Pharbitis* and pea buds (Table II), and in young leaves and cotyledons of amaranth, fitting the general correlation found in animal, bacterial, and plant systems (6, 10, 26).

Other HPLC methods have been described for polyamine analysis, involving separation through ion-exchange columns and post-column derivatization with *o*-phthalaldehyde (1) or ninhydrin (11). The disadvantages of these systems are long analysis time, relatively low sensitivity, and laborious extraction and purification procedures (11). In contrast, the reverse-phase HPLC system reported here is suitable for direct determination of polyamines in crude extracts, with good recovery and short (15–20 min) analysis time. No instability problems are observed with the reverse-phase columns, as opposed to the silica partition or resin ion-exchange columns. Elaborate regeneration procedures are unnecessary after each injection. Optimal column performance is regained after flushing with 100% organic solvent, and more than 100 analyses can be performed before regeneration is needed. Although dansylpolyamines have been determined by combined reverse-phase ion pair HPLC (23, 24), longer analysis time is also required, and the same drawback mentioned above for TLC of dansylpolyamines applies in this latter system. Although polyamine titers in plant tissues are usually at least two orders of magnitude higher than those of plant hormones, the sensitivity of the method described here is in the range already found for HPLC of IAA,

ABA, and cytokinins (21). Considering the evidence involving polyamines in growth regulation (3, 7, 8, 10, 14, 19), it would be desirable to develop methods for simultaneous determination of polyamines and plant hormones.

The procedure we have described is designed for the analysis of free polyamines, operationally defined as those extracted with acid from plant cells, and presumably bound only electrostatically to cell components. Polyamines that may be tightly bound to lipid domains are likely to be excluded from this extraction. Covalently bound polyamines occur as acetyl derivatives in animal systems (22, 24) and as phenolic and other conjugates in plants (5). Little is known about the titer of these conjugates during growth and development, and since they may account for a substantial fraction of the polyamine pool, development of a rapid and precise method for their estimation is important. We now have evidence for the presence of acetylated Put and other polyamine conjugates in both the HClO<sub>4</sub> soluble and insoluble fractions of amaranth extracts (H. E. Flores, unpublished data). The development of an HPLC method for polyamine conjugates in plants is under way. The method reported here, capable of automation, may prove useful for the analysis of crude extracts in a wide variety of higher plants.

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